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Kinetics of biomarkers: biological and technical validity of isoprostanes in plasma

Review Article

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Summary. Isoprostanes, non-enzymatic peroxidation products of arachidonic acid, are attractive biomarkers of oxidative stress in research in biology, medicine and nutrition. For the appropriate use of biomarkers it is required that these are both biologically and technically valid. Whereas the biological validity of isoprostanes is well-established, it is technically quite complicated to measure isoprostanes and its metabolites in body fluids, and its rapid disappearance from plasma may hamper practical application. This paper shortly introduces isoprostanes as a biomarker for studies with humans, describes a novel fast and sensitive method for measuring isoprostanes in plasma by high-performance liquid chromatography and tandem mass spectrometry, and provides several examples of the use of the method in studies in humans. By taking care of the biological and technical validity of this biomarker it is possible to establish the antioxidant effects of some food ingredients in studies with human volunteers.

Keywords: Biomarkers – Isoprostanes – Solid phase extraction – LC-MS-MS – Plasma – Food sciences

Introduction

Over the past two decades consumers' interest in healthy eating shifted from avoiding negatives in foods (e.g., calories, fat, sodium) towards seeking positives (i.e. beneficial nutrients and ingredients).

Recently, the WHO has developed a Global Strategy on Diet, Physical Activity and Health, which was endorsed by the May 2004 World Health Assembly (http://www.who.int/dietphysicalactivity/strategy/eb11344/en/).

This has motivated the food sector to steer towards strategies compatible with public health needs. Therefore, nutrition is at the forefront of the fight against non-communicable diseases, such as obesity, diabetes, cardiovascular disease, and some forms of cancer. In addition, nutrition can significantly improve health status in developing countries where under-nutrition and malnutrition are still evident.

These developments form the basis for a new segment of the food industry: Functional Foods, which can be defined as ''foods and beverages with claimed health benefits based on scientific evidence'' (Diplock et al., 1999; Weststrate et al., 2002). They have triggered considerable efforts in research aimed at finding active ingredients, producing products to be tasty and easy to incorporate in the consumer's preferred lifestyle, and last but not least, providing the scientific evidence. The latter is relevant, as there is general consensus among scientists and regulators that health claims on foods should be substantiated. This is at the interest of authorities and consumers and contributes to fair trade. As such, many national and supranational bodies are currently developing and issuing Codes of Practice to allow claims on foods (Richardson et al., 2003). Whereas most Codes of Practice only contain procedural aspects, the International Life Science Institute is currently finalizing a large EU-sponsored project, Process for the Assessment of Scientific Support for Claims on Foods (Passclaim; http://europe.ilsi.org/passclaim/), which defines a set of criteria for the scientific substantiation of health claims on foods.

In assessing the efficacy of functional foods, much emphasis is dedicated to the role of biomarkers. Biomarkers are the necessary tools to assess efficacy of functional foods and food components, in situations where it is not possible for practical, scientific or ethical reasons to

measure an endpoint directly (Aggett et al., 2005; van Poppel et al., 1997). For the use of biomarkers it is accepted (Aggett et al., 2005) that these need to be both biologically and methodologically valid.

As concerns the latter (methodological validity for biomarkers), the technical variation of the method of analysis is covered by quality control systems in the lab (c.f., e.g., Aggett et al., 2005; Shah et al., 1992; Bressolle et al., 1996).

For this purpose, chemical societies and (inter)national committees for analytical validation give guidance to establish good quality-controlled methods in the laboratory (e.g., www.aoac.org, www.nmkl.org, www.ich.org. and ISO norm 17025; http://www.iso.org/iso/en/ISOOnline.frontpage).

As concerns the former (biological validity), the situation is more complex. In fact many biomarkers are deemed not appropriate (Mensink et al., 2003; Saris et al., 2003; Prentice et al., 2003; Riccardi et al., 2004; Rafter et al., 2004; Westenhoefer et al., 2004; Cummings et al., 2004; Crews et al., 2001a, 2001b; Branca et al., 2001). For example, in the antioxidant area many biomarkers have been described in the literature, but only a handful has been considered acceptable for use in human intervention studies (Griffiths et al., 2002). An essential physiological requirement for the biologically valid use of biomarkers is that they have sufficient sensitivity and specificity (Aggett et al., 2005; van Poppel et al., 1997). Sensitivity and specificity of biomarkers is dependent on inter- and intra-individual differences among individuals. Current developments in the omics area (genomics, transcriptomics, proteomics, and metabolomics) will allow for more insight in the nearby future in inter-individual variation, due to genotypic or phenotypic differences among individuals, and probably also intra-individual variation (Muller and Kersten, 2004).

In addition, there is an extra aspect contributing to variation in biomarkers that need to be taken into account: 'kinetics of biomarkers'. Kinetics of biomarkers is distinct from technical variation and from inter-individual differences, but forms part of biological validation. Kinetics of biomarkers comprises aspects similar to pharmaceutical's kinetics, circadian rhythm, day-to-day variation, etc. (Verhagen et al., 2001, 2004).

To visualise 'kinetics of biomarkers' we can look into the example of cholesterol as biomarker. Cholesterol, a well-established biomarker for cardiovascular disease, is produced in the liver and a smaller extent is coming from the diet. The liver synthesizes the endogenous cholesterol via the enzyme HMG-CoA reductase, which is rate limiting, however, the HMG-CoA reductase enzyme activity is not constant over time, but peaks during the night. Hence, plasma cholesterol concentrations are subject to a diurnal cycle. This should be realised upon designing sampling schemes in human or animal trials. On top of that, the intake via food is also not constant, but is dependent on the average diet. This will change over the year; intake levels during spring and summer with higher fruit and vegetable consumption are assumed to be lower in cholesterol. This has an impact on average plasma concentrations. Thus, the 'kinetics' of the biomarker cholesterol dictates the sampling scheme to correct for the short-term kinetic cycle, but also requires to use parallel control group study designs to adjust for long-term cycle (seasonal) variations (Bluher et al., 2001).

Thus for the appropriate use of a biomarker both the technical and methodological variation needs to be taken into account. This will be further illustrated in the present paper for the oxidative stress biomarker ''F2-alpha-isoprostanes''. F2-isoprostanes are an accepted biomarker for oxidative stress (Griffiths et al., 2002) thereby supporting its biological validity. In particular this paper reports on a method for improved technical measurement of F2-isoprostanes, and shows it practical application in a kinetics study and use in human intervention studies.

Isoprostanes as biomarkers of oxidative stress

Free radicals are known to be involved in ageing and age-related diseases (Emerit and Chance, 1992; Harman, 1992; Sohal and Brunk, 1992; Bondy, 1992). However it is difficult to determine oxidative damage to tissue macromolecules (DNA, proteins, lipids), originating from free radicals. This is especially true for the determination of biomarkers of oxidative damage in healthy humans (Griffiths et al., 2002; Crews et al., 2001). As concerns the latter, isoprostanes are considered to be one of the best biomarkers for tissue macromolecule oxidation caused by oxidative stress (Griffiths et al., 2002). Isoprostanes (Crews et al., 2001b; Roberts and Morrow, 2000; Pratico, 1999; Lawson et al., 1999) were extensively characterised by Morrow et al. (1992, 1998) as free radical mediated oxidation products of arachidonic acid, e.g., 15-F2t-Isoprostane (F2aIP), 8-iso-15(R)-prostaglandin F2α, 8-iso prostaglandin F2β.

The free radical product that is used most frequently as a biomarker for oxidative stress is (F2aIP) (Fig. 1). However, the kinetics of this biomarker needs to be taken into account as (F2aIP) in plasma has a short half-life time (measured in rabbits: approximately 18 min. Moreover

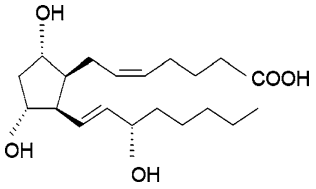

Fig. 1. Chemical structures of 15-F2t-isoprostane

F2aIP is further metabolised into 2,3-dinor 8-iso PGF1a in urine (Morrow et al., 1990, 1999). Also technically there are issues, and some limitations with respect to their measurement have to be taken into account. While the total plasma level of (F2aIP) is about 150 pg in normal volunteers, most of the isoprostanes in plasma are present partly in the esterified form, and partly in the free form (ratio 2:1). This requires a hydrolysing step in the analytical procedures as well as the need for a highly selective and sensitive analytical technique for the determination in biological fluids.

Analysis of isoprostanes in plasma

Until recently most laboratories have used GC-MS or GC-MS-MS as the analytical separation and detection method for (F2aIP) (Basu, 1998; Mori et al., 1999). Though these methods have advantages of high specificity and sensitivity, the major disadvantage is the laborious sample preparation and the prerequisite of derivatization (to make the analytes volatile) required for the GC separation (Schweer et al., 1997; Morrow and Roberts, 1994). Recently, HPLC combined with a mass spectrometer (HPLC-MS or HPLC-MS-MS) has became more and more available in many laboratories involved in biological research. The advantage of HPLC compared to GC is its potential to measure less volatile compounds without the requirement of a derivatization step. This leads to a less complicated and less error-prone sample preparation method and to a higher throughput of the number of samples. Some early reports on the analysis of isoprostanes by HPLC (MS) have been published (Walter et al., 2000; Murai et al., 2000). We have taken this further and in the paper presented here, as an example, a validated, specific and sensitive (18 pg/mL plasma) HPLC-MS-MS methodology for the determination of isoprostanes in plasma is described.

Methodological aspects

In addition to normal reagent grade chemicals, (F2aIP), (F2aIP)-D4, 8-iso-15(R)-prostaglandin F2α, 8-iso prostaglandin F2β, 5-trans Prostaglandin F2α, Prostaglandin 2α, and 11β-Prostaglandin F2α were obtained from Cayman Chemicals (Ann Arbor, Michigan, USA); 5,6,8,9,11,12,14,15(n)-3H (F2aIP) was obtained from Amersham (Amersham Bioscience, Piscataway, NJ, USA). In short, to 1000 μL thawed and homogenised plasma was added: 30 μL internal standard (300 pg deuterated (F2aIP)), 80 μL of a solution containing BHT (10 mM) and triphenylphosphine (1 mM) in ethanol, and the mixture was vortexed thoroughly. Then 400 μL of a solution containing 25% (w/v) KOH was added and the mixture was heated on a water bath for 45 minutes at 40°C in order to hydrolyse esterified isoprostanes. After hydrolysis the sample was diluted with 3 mL water (in order to prevent protein precipitation) followed by vortexing. Acetic acid was added to pH 3 to prevent dissociation of the isoprostanes. A solid phase column (tC18 Sep-pak Vac, 3 mL, 200 mg; Waters Chromatography, Etten-Leur, The Netherlands) was conditioned with 3 mL methanol, followed by 3 mL de-ionised water that was adjusted to pH 3 with glacial acetic acid. The sample was vortexed again and loaded onto the SPE column. The test-tube was washed with 3 mL water (pH 3, corrected with glacial acetic acid) and the washing solution was also loaded onto the column and cartridge was dried. The cartridge was then washed with 3 mL heptane to remove lipids. The cartridges were dried again under vacuum after which the sample was eluted with 7 mL of eluent consisting of ethyl acetate/heptane/methanol (50/40/10%). The resulting solution was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 60 μL acetonitrile/water (60%/40% with 0.05% (v/v) acetic acid). The sample was filtered using micro spin filter tubes (0.2 μm nylon, Alltech Associates Inc., Deerfield, USA) on a IEC Micromax RF micro centrifuge, at 12000 rpm.

In order to determine the recovery, plasma spiked with radioactive labelled 5,6,8,9,11,12,14,15(n)-^{3}H-F2t-isoprostane was used (51.9 pg/mL). The total activity before and after the hydrolyses/SPE procedure was measured on a Packard Tri-Carb 1900CA (Packard Bioscience, Meriden) and the recovery was calculated from the ratio of the counts after and before the sample preparation procedure. A reversed-phase HPLC C18 Symmetry column (dp 3.5 μm) 150 × 2 mm I.D. (Waters Chromatography B.V., Etten-Leur, The Netherlands) was attached to a Waters Alliance 2790 liquid chromatographic system (Waters Chromatography B.V., Etten-Leur, The Netherlands). The HPLC system was connected to a Micromass Quatro Ultima Mass Spectrometer (Micromass, Manchester, UK) with an electrospray interface.

A gradient separation was performed starting with a solvent that consisted of 60% water (pH 3.85) and 40%

acetonitrile running for 4 minutes. In 1 minute a gradient was run to 100% acetonitrile which ran till 8 minutes. From 8 to 9 minutes a gradient was used to return to the original eluent composition and the system was equilibrated till 15 minutes. The separation takes place in the autocratic part of the chromatogram. The gradient was used to remove matrix compounds fast from the column leading to a faster next injection. In order to investigate if the *in vivo* metabolite of Prostaglandin D2, 11β-Prostaglandin F2 was disturbing the analyses of the F2aIP the composition of the mobile phase was changed to 35% acetonitrile during the isocratic period of the separation. The flow rate was 200 μL/min, the temperature was set at 20°C and the injection volume was 30 μL. The samples were analysed in the negative multiple reaction mode of the MS. In the MRM (multiple reaction monitoring) mode the dwell time was 400 ms; the pause time was 100 ms. Daughter spectra of the synthetic compound and the internal standard were recorded via direct infusion of a solution in water (200 pg/μL). MCA (multiple count analysis) scans were recorded, the scan time was 5 seconds, the pause time was 100 ms. Target ions were selected at m/z 353/193 for isoprostanes and at m/z 357/197 for the deuterium labelled internal standard. The mass spectrometer was set to the following optimised parameters: Capillary voltage 3 kV, cone voltage 70 V, collision energy 25 eV, source temperature 120°C, desolvation temperature 200°C, cone gas 175 L/h (nitrogen), desolvation gas 525 L/h (nitrogen). Nitrogen was used as the collision gas.

Technical validation of the analysis of isoprostanes in plasma

The daughter spectra of the synthetic compound of (F2aIP) and the internal standard were described before in literature by others (Ohashi an Yoshikawa, 2000; Murai et al., 2000). The analyses require complete hydrolysation of the esterified form of the F2-isoprostane what was achieved after 30 minutes (see Fig. 2). Figure 3a shows the chromatograms of the standard solution of (F2aIP) and its deuterated standard: this shows that the retention time of the isoprostanes is 4.1 min. Figure 3b shows a chromatogram of pooled plasma spiked with the internal standard. It can be seen that F2aIP is separated from the other compounds.

Spiking plasma with the primary metabolite of PGD2 which is present *in vivo* in urine, 8β-prostaglandin F2α showed no baseline separation. This metabolite has not been described in the literature in plasma. To be sure that there is no interference with the commercially available

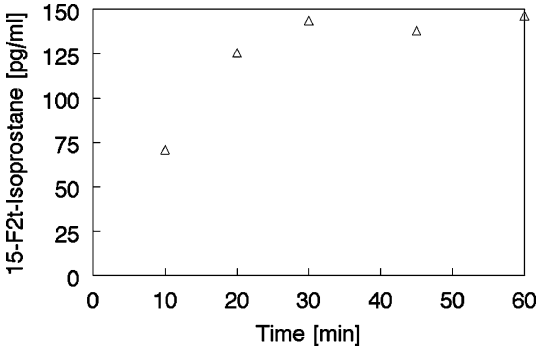

Fig. 2. The amount of free isoprostanes formed versus time of hydrolysis of plasma

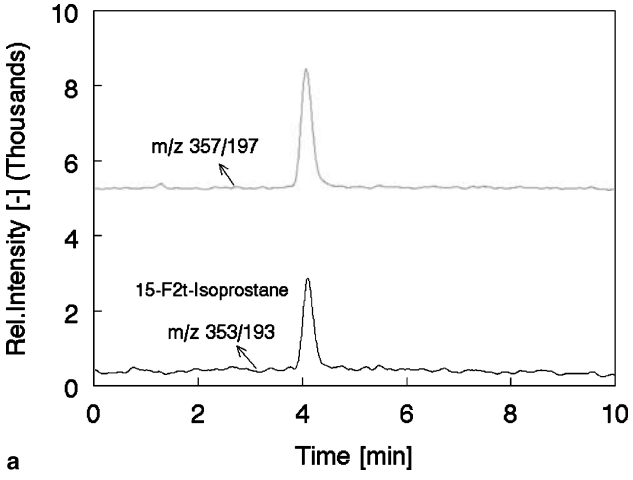

Fig. 3a. HPLC-MS-MS chromatograms of a mixture of synthetic 15-F2t-isoprostane and its deuterated internal standard (IS) (both 50 pg/mL) in mobile phase

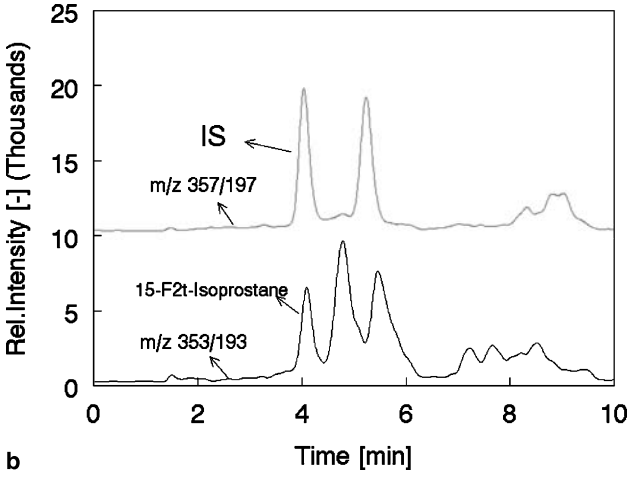

Fig. 3b. HPLC-MS-MS chromatograms of 15-F2t-isoprostane in plasma and its deuterated internal standard (IS)

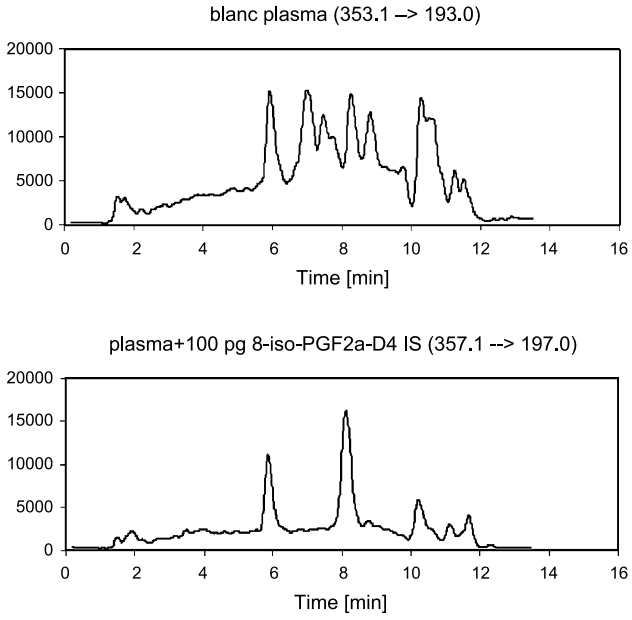

Fig. 4. HPLC-MS-MS chromatograms of 15-F2t-isoprostane compared to plasma spiked with its deuterated internal standard (IS)

isoprostanes and the metabolite, plasma was spiked separately with each standard and with the metabolite. The mobile composition was changed to 35% acetonitrile to increase the separating power resulting in a longer retention time. Only three commercial available isoprostanes co-elute, however, this is not a problem since they are all markers for oxidative damage (O'Sullivan et al., 1996). The results with 35% acetonitrile also show that a separation was achieved between the isoprostanes and the metabolite. In the blanc plasma no metabolite could be detected meaning that the metabolite will not interfere with the peaks of the isoprostanes in the original chromatogram (Fig. 3b). Figure 4 shows the chromatograms of blanc plasma and plasma spiked with F2aIP.

The intermediate precision $S_{I(T)}$ was 9%, determined using pooled plasma that was measured 5 times (in one day) on 4 different days. ANOVA analyses showed that there was no difference between the within day variability and the between day variability. The recovery was determined comparing the activity of a radiolabeled solution (in plasma) before and after the sample preparation (hydrolysis, SPE) was used on the plasma sample. The recovery was 98%. Since the chromatographic behaviour of the isoprostanes investigated, under these conditions are the same, the same recovery can be assumed the same for 8-iso-15(R)-prostaglandin F2α and 8-iso prostaglandin F2β. The accuracy was determined by spiking a solution of plasma with 5 different concentrations of (F2aIP). The determined concentration was linear plotted against

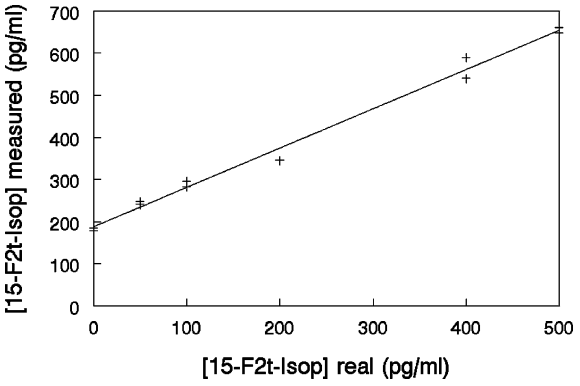

Fig. 5. Concentration of F2aIP in plasma determined with HPLC-MS-MS plotted against real concentration. No proportional bias was found. $R^2 = 0.9922$ (experimental conditions are as described in the materials and methods section)

the actual concentration ($y = 0.931 \times$ [15-F2t-isoprostane real] + 188.73) (see Fig. 5). The 95% confidence interval for the intercept was determined as $186.20 < a0 \leq 210.71$; the 95% confidence interval for the slope was $0.857 < a \leq 1.000$ (corrected after subtraction of the blanc value). Since the slope should be 1, a value that fits into the confidence interval no proportional bias was found. The accuracy ranged from 2.9–16%. The calibration curve was linear at least until 1000 pg/mL ($R = 0.995$).

The equation of the calibration curve is: area (isoprostanes)/area((F2aIP)-D4) $= 0.0301 \times$ [isoprostanes] 0.1104.

The limit of quantification in plasma (LOQ) ($\alpha = 0.05$) was determined according to the IUPAC model ({HYPERLINK http://www.iupac.org/dhtml_home.html}) as 18 pg/mL plasma. On 16 different days 4 identical samples of pooled plasma from human volunteers were measured and plotted as a Shewhart Chart (see

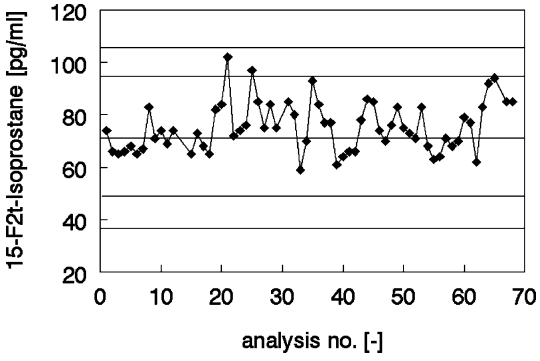

Fig. 6. Shewhart Chart of the sum of free and esterified isoprostanes. The concentration of pool plasma determined on several days (4 samples per day) is plotted against the analysis number. The lines in the plot represent the mean value, the mean $\pm$ 2SD and the mean $\pm$ 3SD (experimental conditions are as described in the materials and methods section)

Fig. 6). Two samples were outside the range mean ± 2SD, but not in two upfollowing runs, meaning that quality criteria that 95% of the analyses have to be in the normal range have been reached.

Application of a biologically and technically validated biomarker

Whereas isoprostanes are biologically well-established as a biomarker for oxidative damage to lipids *in vivo* (Griffiths et al., 2002), we have shown above that also a technically validated and versatile method is available. The method above is sufficiently selective and sensitive to measure total (i.e. free and esterified) isoprostanes levels in plasma and to be applied in *in vivo* studies with humans.

1. Kinetics of F2aIP

Claudicants are know to be exposed to oxidative stress during exercise. In this study we looked at the kinetics of the formation of isoprostanes in plasma in time during an exercise protocol. Patients suffering from claudicatio intermittent disease exercised for 5 minutes. Blood was sample before exercise (5 min period) and 4 points after exercise. The influence of supplementation of vitamin E (200 mg/day) and C (1000 mg/day) for four weeks, was also investigated. As Fig. 7 clearly shows, directly after the exercise an increase in the levels of isoprostanes is seen. This is the reperfusion period where most of the oxidative stress is expected. As can be seen after two hours the concentration of isoprostanes is back to it base level during resting meaning that the choice of time of measurement easily can lead to erroneous conclusions.

2. Influence of a spread enriched with vitamin E and carotenoids on F2-isoprostanes

The method was used in a clinical study where the influence of vitamin E and carotenoids in a margarine spread was tested in a human trial. A randomised, controlled, double-blind, 11-week parallel intervention study was used to examine the effect of moderate doses of a combination of vitamin E and carotenoids, incorporated into a food product, on markers of antioxidant status and lipid peroxidation in healthy people. After 2-weeks stabilization on a commercial non-fortified spread, subjects consumed 25 g/d of spread containing either: 43 mg a-tocopherol equivalents (a-TE) (2–3 fold US DRI level) and 0.45 mg carotenoids (A), n = 35; 111 mg a-TE and 1.24 mg carotenoids (B), n = 35; or a non-fortified control spread (2.3 mg a-TE) (C), n = 35. Both spread A and spread B successfully raised plasma a-tocopherol concentrations to greater than 30 mmol/L and produced small but significant increases in a-carotene and lutein concentrations. Spread B significantly reduced (15%) the plasma lipid peroxidation biomarker. The concentration of the Isoprostanes found was in the order of 80 pg/mL plasma (Upritchard et al., 2003). The data in Fig. 8 demonstrate plasma total $F_{2\alpha}$-isoprostane concentrations are stable in healthy human volunteers over several weeks. Individuals changed less than 5% (NS). In contrast, giving an antioxidant supplement such as vitamin E (100 mg/d) resulted in a reduction in plasma total $F_{2\alpha}$-isoprostane concentra-

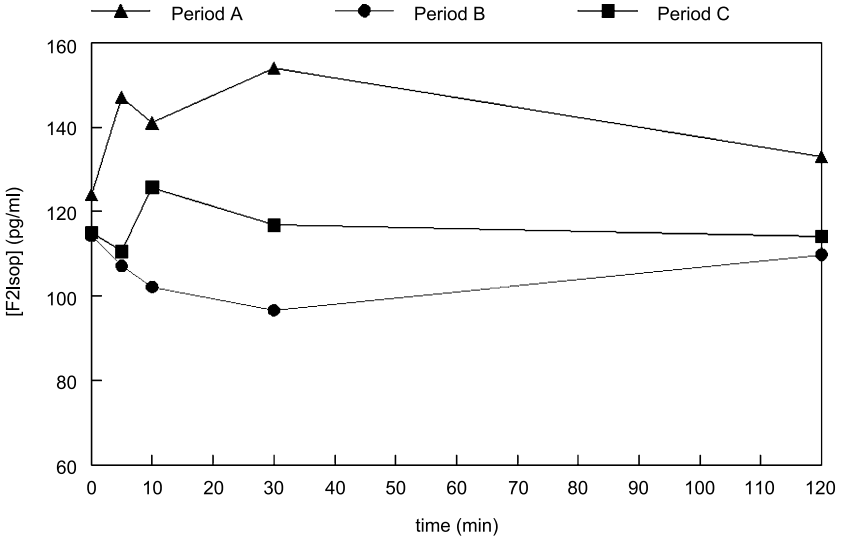

Fig. 7. Concentration of F2aIP in plasma of patients during exercise (0–5 min) and the recovery period after the exercise (5–120 min). A, baseline period; B, after 4 weeks administration of 200 mg vitamin E and 1000 mg vitamin C daily; C, after 8 weeks washout period of the vitamins

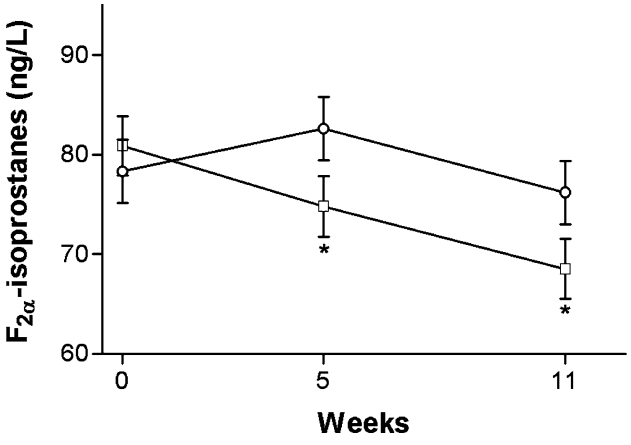

Fig. 8. Circles show the concentration of plasma isoprostanes (ng/L) in healthy volunteers that consumed a placebo product for 11 weeks. Boxes show the reduction in plasma isoprostane (ng/L) concentrations when healthy adults consume 100 mg/d of vitamin E. The reduction is significant within 5 weeks and continued until the end of the study at 11 weeks. $^{*}p < 0.05$

tions of 15% ($p < 0.001$). This shows that the kinetics of the isoprostanes play a less important role if medium/long term effects of antioxidants are studied, this in contrary to a burst of oxidative stress (e.g., exercise).

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